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Gingival tissue transcriptomes in experimental gingivitis

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Abstract

Aims: We investigated the sequential gene expression in the gingiva during the induction and resolution of experimental gingivitis.

Material and Methods: Twenty periodontally and systemically healthy non-smoking volunteers participated in a 3-week experimental gingivitis protocol, followed by debridement and 2-week regular plaque control. We recorded clinical indices and harvested gingival tissue samples from four interproximal palatal sites in half of the participants at baseline, Day 7, Day 14 and Day 21 (the ''induction phase''), and at Day 21, Day 25, Day 30 and Day 35 in the other half (the ''resolution phase''). RNA was extracted, amplified, reversed transcribed, amplified, labelled and hybridized using Affymetrix Human Genome U133Plus2.0 microarrays. Paired *t*-tests compared gene expression changes between consecutive time points. Gene ontology analyses summarized the expression patterns into biologically relevant categories.

Results: The median gingival index was 0 at baseline, 2 at Day 21 and 1 at Day 35. Differential gene regulation peaked during the third week of induction and the first 4 days of resolution. Leucocyte transmigration, cell adhesion and antigen processing/presentation were the top differentially regulated pathways.

Conclusions: Transcriptomic studies enhance our understanding of the pathobiology of the reversible inflammatory gingival lesion and provide a detailed account of the dynamic tissue responses during the induction and resolution of experimental gingivitis.

Key words: experimental gingivitis; gene expression; microarray; tissue response

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Since its inception approximately 50 years ago (Löe et al. 1965), experimental gingivitis has been used extensively as a clinical research tool in the study of the pathobiology of the reversible gingival lesion that was shown to develop in response to the accumulation of dental plaque on the adjacent tooth surfaces. Over the years, a substantial body of data has accrued on the microbiologic features of the early gingival lesion (Moore et al. 1982, 1984), including the influence of gingival inflammation on plaque formation (Hillam & Hull 1977, Loesche & Syed 1978, Daly & Highfield 1996) as well as the differences in microbial profiles between individuals with a high and a low propensity for gingival inflammation (Lie et al. 1995) or between subjects with different levels of susceptibility to periodontitis (Abbas et al. 1986). Other studies have used histology and/or immunohistochemistry to characterize the cellular components of the gingival lesion (Payne et al. 1975, Seymour et al. 1983, Kinane et al. 1991, Fransson et al. 1999) or to identify proteins that are secreted into the gingival crevicular fluid (GCF) during the development and the resolution of gingival inflammation (Lamster et al. 1985, Heasman et al. 1993, Deinzer et al. 2007, Offenbacher et al. 2009a, Grant et al. 2010). Lastly, the experimental gingivitis model has been widely used to study the effects of pharmacological agents that inhibit plaque formation and/or modulate gingival inflammation in humans (Wennström 1988, Jenkins et al. 1993, Ramberg et al. 1995, Quirynen et al. 2001, Sekino et al. 2003, Van Strydonck et al. 2005).

In the past few years, our group has used high-throughput microarray technology in the study of the pathobiology

Conflict of interest and sources of funding statement

The authors declare that they have no conflicts of interest.

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of periodontal diseases and was the first to characterize the whole-genome gingival tissue transcriptomes in different forms of periodontitis and in states of periodontal health and disease (Papapanou et al. 2004, Demmer et al. 2008), as well as to examine the relationship between subgingival microbial colonization profiles and gene expression signatures in the adjacent tissues (Papapanou et al. 2009). In this paper, we extend our previous work and analyse gingival transcriptional profiles concurrent with the induction and resolution of gingival inflammation during the course of experimental gingivitis. We hypothesized that these profiles would be consistent with known elements of the pathobiology of gingivitis, but would also point to the involvement of novel, yet unrecognized molecules and processes. Thus, the aim of the present study was to systematically investigate the sequential gene expression in the gingival tissues that parallels (i) the gradual conversion from a state of pristine periodontal health to a state of established gingivitis and (ii) the resolution of gingival inflammation during re-institution of periodontal health.

Material and Methods

The design of the study was approved by the Regional Ethical Review Board, Göteborg, Sweden (#005-09). Informed consent was obtained from all study participants before enrolment.

Subject sample

A total of twenty, systemically healthy volunteers were recruited among the undergraduate students attending the Faculty of Odontology, Sahlgrenska Academy, Göteborg University, Sweden. All participants were free of interproximal attachment loss and had no probing pocket depths of >4 mm. Buccal recessions of obvious traumatic aetiology at single teeth did not automatically disqualify a volunteer from participation. The participants were non-smokers, were not current users of antibiotics, contraceptives or immunosuppressive drugs, and were not pregnant or lactating. They were assigned to one of two groups: a ''gingivitis induction'' and a ''gingivitis resolution'' group comprising 10 individuals each, equally many female and male. With respect to other demographic characteristics, 19 individuals were Caucasian while a single female participant in the resolution group was Asian. The mean age was 24.7 years in the induction group (median 21 years, range 20–31) and 24.4 years in the resolution group (median 24, range 21–29).

Experimental gingivitis protocol

During a 3-week preparatory period before the experimental gingivitis phase, all volunteers were instructed in proper oral hygiene measures (tooth brushing and interproximal cleaning using dental floss) and were subjected to between two and three sessions of professional tooth cleaning using a rubber cup and polishing paste until they showed no or only minimal signs of gingival inflammation (average full-mouth gingival index (GI) (Löe 1967) <0.2). Maxillary impressions were obtained and acrylic stents that covered the palatal gingival tooth surfaces of all maxillary teeth were fabricated. After the establishment of absence of gingival inflammation, experimental gingivitis was induced over a 3-week period at the maxillary palatal surfaces. During this time, the participants were asked to abstain from brushing of the palatal surfaces of the maxillary arch and from any means of interproximal cleaning. To prevent accidental removal of plaque from the experimental sites, the individually fabricated stents were always put in place during the regular brushing of the maxillary buccal surfaces and the mandibular teeth. After completion of the 3-week gingivitis induction phase, all participants received thorough oral prophylaxis by the same dental hygienist, including full-mouth debridement and polishing. Oral hygiene measures including tooth brushing and dental flossing at least twice daily were reinstituted in the entire dentition. The ''gingivitis resolution'' phase was completed 2 weeks after re-institution of regular oral hygiene.

Clinical assessments

GI (Löe 1967) assessments were carried out bilaterally at the mesio-palatal and the disto-palatal aspects of each inter-dental papilla between the first and second maxillary premolars and between the first maxillary premolar and canine, using a periodontal probe, by a single calibrated examiner (author P. R.).

Bacterial plaque samples and processing

Immediately after the clinical assessments, a sterile paper point was inserted at the mesio-palatal and disto-palatal aspects of each of the above inter-dental papillae, left in place for 30 s, and transported in sterile Tris-EDTA buffer. The plaque samples were analysed individually using the checkerboard DNA–DNA hybridization method (Socransky et al. 1994, Papapanou et al. 2001) as described earlier with respect to the following 18 bacterial species: *Porphyromonas gingivalis*, *Tannerella forsythia*, *Treponema denticola*, *Prevotella nigrescens*, *Prevotella intermedia*, *Parvimonas micra*, *Fusobacterium nucleatum*, *Campylobacter rectus*, *Capnocytophaga ochracea*, *Streptococcus sanguis*, *Streptococcus mutans*, *Streptococcus intermedius*, *Streptococcus oralis*, *Actinomyces naeslundii*, *Veillonella parvula*, *Selenomonas noxia*, *Eikenella corrodens* and *Aggregatibacter actinomycetemcomitans*.

Collection of gingival tissue samples

As mentioned above, to minimize the number of sequentially obtained tissue samples per participant to a maximum of four, we studied the induction of gingivitis separately from the resolution of gingival inflammation in two distinct groups of patients. After local infiltration anaesthesia with 2% lidocaine-HCl 2% with 1:100,000 epinephrine, gingival tissue samples amounting to approximately 8 mm^3 and comprising both the sulcular epithelium and the underlying connective tissue were obtained from the palatal aspects of four interproximal papillae in the following sequence: the papilla between the upper canine and the first premolar, followed by its contra-lateral site, followed by an interproximal papilla between the two upper premolars, followed by its contra-lateral site. These biopsies were obtained at the following time points: in the induction group, at baseline (Day 0), and at the completion of the first week (Day 7), second week (Day 14) and third week (Day 21) of experimental gingivitis. In the resolution group, biopsies were obtained at the completion of 3 weeks of experimental gingivitis (Day 21), and at 4 (Day 25), 9 (Day 30) and 14 days (Day 35) after the provision of full-mouth prophylaxis and re-institution of oral hygiene procedures.

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Gingival tissue processing

Immediately after harvesting, each tissue sample was rinsed with sterile saline and placed in an individually labelled Eppendorf tube with an RNA stabilizing agent (RNAlater, Ambion Inc., Austin, TX, USA). The biopsies were held at 4°C overnight in RNAlater, the liquid was subsequently decanted and the tube was snap-frozen in liquid nitrogen. The samples were held at −70°C until being shipped to the laboratory at the Division of Periodontics, Columbia University, in a single batch, in dry ice. The transportation time did not exceed 24 h and all tissue samples were frozen upon arrival.

Isolation of total RNA, reverse and in vitro transcription, labelling and hybridization

We largely followed the protocol recently described in detail by Kebschull & Papapanou (2010). In brief, the tissue specimens were homogenized in TRIzol (Invitrogen Life Technologies, Carlsbad, CA, USA) and total RNA was isolated and purified using RNeasy cleanup columns (Qiagen, Valencia, CA, USA). RNA quantity and quality were evaluated in all cases spectrophotometrically using a NanoDrop 1000 device (Thermo Scientific, Wilmington, DE, USA). In preparatory experiments, sample quality was assessed using an Agilent 2100 Bioanalyzer and RNA Nano Chips (Agilent Technologies, Santa Clara, CA, USA), consistently demonstrating RNA integrity numbers $\geqslant 9$. One hundred nanograms of total RNA was reverse- and in vitro transcribed, labelled and fragmented using the 3′ IVT kit (Affymetrix, Santa Clara, CA, USA), and 15 μg of the labelled RNA was hybridized using a Human Genome U133 Plus 2.0 Array (Affymetrix), which carries >55,000 probe sets mapping to approximately 38,500 well-characterized genes.

Data analysis

Gene expression data were analysed as described previously (Demmer et al. 2010). In brief, Affymetrix array data were first normalized and summarized using the log-scale robust multi-array analysis (Irizarry et al. 2003) with default settings. For each probe set, a fold change was computed by dividing the mRNA expression value at each time point by the expression value of the immediately preceding time point, or to baseline, i.e., Day 0 in the ''induction'' group and Day 21 in the ''resolution'' group. *p*-values from the aforementioned analyses were input into gene ontology analysis using the Pathway Express software (Draghici et al. 2007, Khatri et al. 2007) to identify biologically relevant groups of genes that showed changes in expression over time. Gene symbols and descriptions were downloaded from http://www.bioinformatics.ubc.ca/microannots/

Results

Clinical findings

Figure 1 illustrates the development and resolution of experimental gingivitis reflected through the GI scores at the experimental sites. The mean GI was 0.1 at Day 0 (median 0), and increased to 0.7 at Day 7 (median 1), to 1.0 at Day 14 (median 1) and peaked at 1.6 (median 2, range 1–2) at Day 21. In the resolution phase, the average GI was 1.8 (median 2, range 1–2), with 2 of the 10 participants showing a GI of 1 after 3 weeks of experimental gingivitis induction). The average GI was reduced to 1.4 at Day 25 (median 1), to 0.8 at Day 30 (median 1) and at 0.7 at Day 35 (median 1). Only 3 participants had a GI score of 0 at the end of the resolution phase, with the remaining 7 showing a GI of 1.

Microbiological findings

Supporting information Fig. S1 describes the bacterial colonization profiles at the gingival crevices adjacent to the harvested gingival tissue samples during the induction and resolution phases. Levels of the ''red complex'' species *P. gingivalis*, *T. forsythia* and *T. denticola* were much lower than those of all other investigated bacteria throughout both the phases. A conspicuous increase in certain ''orange complex'' bacteria including *P. nigrescens* and *P. intermedia*, and to a lesser extent *P. micra*, was observed during gingivitis induction, and the levels of these species declined during the resolution phase. Likewise, streptococcal spp. and levels of *Actinomyces naeslundii*, which dominated the microbial profiles, showed a similar pattern of increase and decline during the induction and resolution phases, respectively.

Transcriptomic responses

Dynamics of the sequential gene activation

To initiate the analysis of the sequential activation of genes over time, we first explored the number of probe sets that were statistically significantly ($p<0.05$) differentially regulated between any two consecutive time points. In the induction phase, 5278 probes were differentially expressed ($p<0.05$) during the first week (i.e., Day 7 *versus* baseline); 3660 probes in the second week (Day 14 *versus* Day 7); and 6765 probes during the third week (D21 *versus* D14). A comparison between Day 21 and baseline yielded a total of 3170 differentially regulated probes. In the resolution phase, 7250 probes were significantly differentially regulated during

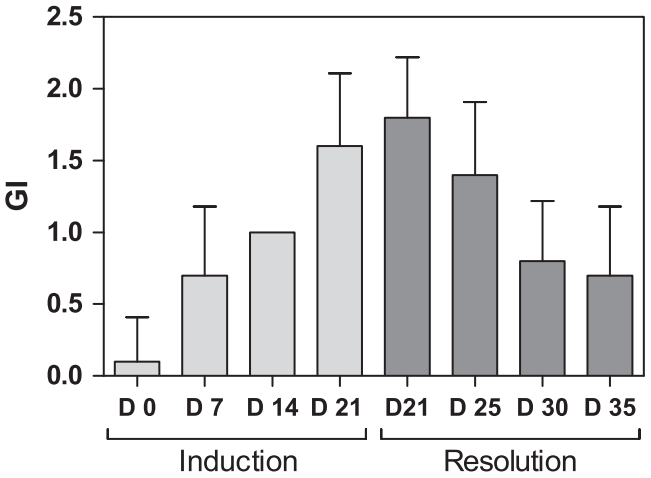

Fig. 1. Gingival index at the experimental sites during the induction and resolution of experimental gingivitis. Bars represent the means and standard deviations.

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the first 4 days after prophylaxis and re-institution of oral hygiene (i.e., Day 25 *versus* Day 21); 5085 probes in the next 5 days (Day 30 *versus* Day 25); and 2698 probes during the final 5 days (D35 *versus* D30). A comparison between Day 35 and Day 21 yielded 7763 differentially regulated probes.

Subsequently, we examined the number of probes with an absolute fold change of >1.5, i.e., probes that were either upregulated or downregulated by at least 50%, between two consecutive time points. In the Induction phase, a total of 127 probe sets were differentially regulated between baseline and Day 7 by >1.5-fold (Fig. 2). Of these, 85 were upregulated and 42 were downregulated. During the second week of gingivitis induction, only three probe sets were upregulated while 71 were downregulated. Differential gene expression was maximized during the third week of induction (between Day 21 and Day 14), with a total of 373 probe sets being differentially regulated >1.5-fold, 81% of which (301 probes) were upregulated and 19% (72 probes) were downregulated. A comparison between the time point of maximal inflammation (Day 21) and baseline yielded a total of only 184 differentially regulated probe sets, i.e., less than half of the number found to be differentially regulated during the third week of induction alone.

The bottom panel of Fig. 2 provides the corresponding description of sequential gene activation during the Resolution phase. It is evident that most of the activity in the gingival tissues in terms of differential gene expression occurred within the first 4 days post-prophylaxis and re-institution of oral hygiene. Out of a total of 470 probe sets that were statistically significantly differentially expressed with an absolute fold change of 1.5, 93% (439 probes) were downregulated and only 7% (31 probes) were upregulated. In comparison, far fewer probe sets were differentially expressed during the subsequent time intervals: 118 between Day 30 and Day 25 (69% downregulated), and only 24 between Day 35 and Day 30. A comparison between the first and the last points of the resolution phase showed a total of 367 differentially expressed probe sets, in their vast majority (89%) downregulated.

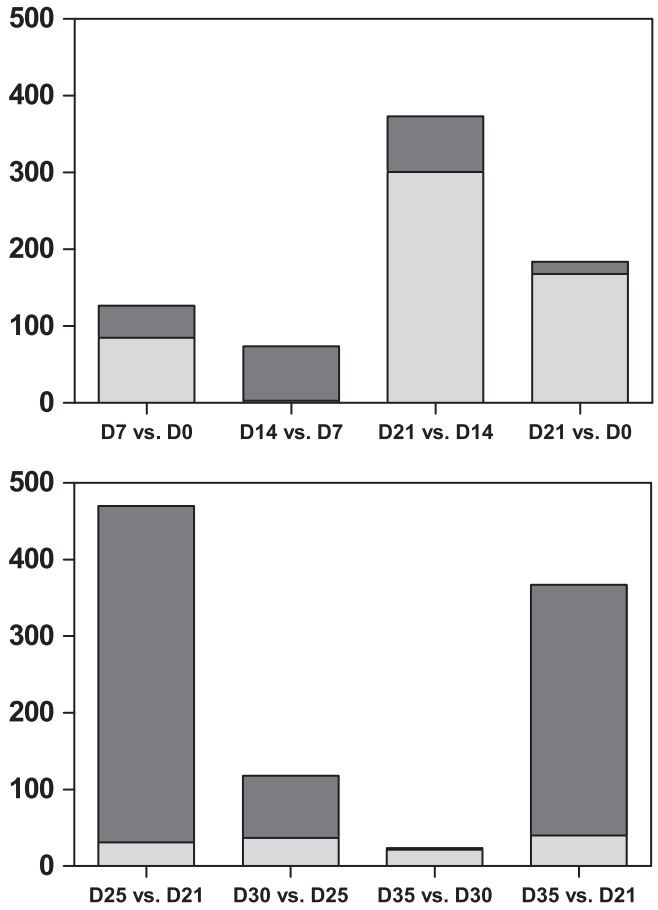

Fig. 2. Number of differentially expressed probe sets with an absolute fold change of >1.5 during the induction (top) and resolution phases (bottom). Light grey bars indicate upregulation, dark grey bars downregulation.

Tables 1 and 2 list the top 20 probes that mapped to annotated genes and were found to be differentially expressed during the induction and resolution phases, respectively. In these tables, the depicted fold changes in expression were based on data from two consecutive time points and were calculated as the ratio of expression at the latter time point over that of the former time point (i.e., Day 7/Day 0, Day 14/Day 7 and Day 21/14 in the induction phase, Table 1a–c; and Day 25/Day 21, Day 30/Day 25 and Day 35/Day 30, in the resolution phase Table 2a–c). In both Tables, probes are sorted according to descending absolute fold change, i.e., according to decreasing magnitude of differential regulation irrespective of direction (up- or down-regulation). Complete lists of all differentially regulated probes between any two consecutive time points along with the corresponding fold changes and *p*-values are provided in the supporting information Tables S1–S6.

Gene ontology analyses

Using the Pathway Express software, we summarized the acquired expression profiles into biological processes. Tables 3 and 4 list the top five differentially regulated pathways between each pair of consecutive time points in the gingivitis induction and resolution phases, respectively. The Tables also list the total number of genes included in each pathway; the percentage of genes in the particular pathway that were statistically differentially regulated ($p<0.05$); the impact factor of each individual pathway, which is a probabilistic term that takes under consideration both the proportion of the differentially regulated genes in the pathway and the perturbation of each gene; and finally the *p*-value for the differential regulation of the particular pathway. The top two gene ontology groups in both the first and the second week of gingivitis induction were leucocyte trans-endothelial migration and cell adhesion, while antigen processing and presentation was the top regulated pathway in the third

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Table 1. Top differentially expressed probes mapping to annotated genes, during the *(a)* first week, *(b)* second week and *(c)* third week of gingivitis induction, sorted according to descending absolute fold change

Rank	Gene	Description	Fold change	*p*-value
(a)				
1	CRISP3	Cysteine-rich secretory protein 3	5.54	0.00261
2	MS4A1	Membrane-spanning four-domains, subfamily A, member 1	3.24	0.015318
3	ATP6V0A4	ATPase, H+transporting, lysosomal V0 subunit a4	2.64	0.00379
4	CD177	CD177 molecule	2.54	0.035596
5	CLCA4	Chloride channel, calcium activated, family member 4	2.52	0.032565
6	GYS2	Glycogen synthase 2 (liver)	2.38	0.003151
7	TMPRSS2|AK	Transmembrane protease, serine 2	2.28	0.009112
8	TMPRSS2|AK	Transmembrane protease, serine 2	2.13	0.027892
9	ERO1L	ERO1-like (*S. cerevisiae*)	2.05	0.014052
10	IGFBP6	Insulin-like growth factor 2 mRNA-binding protein 3	0.49	0.010173
11	ERO1L	ERO1-like (*S. cerevisiae*)	1.98	0.017879
12	IGF2BP3	Insulin-like growth factor 2 mRNA-binding protein 3	1.97	0.000065
13	CDKN3	Cyclin-dependent kinase inhibitor 3 (CDK2-associated dual specificity phosphatase)	0.51	0.016633
14	SILV	Silver homologue (mouse)	0.52	0.022768
15	GPR37	G-protein-coupled receptor 37 (endothelin receptor type B-like)	0.52	0.012444
16	PPP1R3C	Protein phosphatase 1, regulatory (inhibitor) subunit 3C	1.92	0.013479
17	C15orf48	Chromosome 15 open reading frame 48	1.91	0.003409
18	CDKN3	Cyclin-dependent kinase inhibitor 3 (CDK2-associated dual-specificity phosphatase)	0.52	0.017647
19	FUT3|FUT5|	Fucosyltransferase 3 (galactoside 3(4)-L-fucosyltransferase, Lewis blood group)|fucosyltransferase 5	1.88	0.010279
20	PHLDA1	Pleckstrin homology-like domain, family A, member 1	1.86	0.027904
(b)				
1	CRISP3	Cysteine-rich secretory protein 3	0.33	0.046981
2	POU2AF1	POU domain, class 2, associating factor 1	0.49	0.043393
3	FAM46C	Family with sequence similarity 46, member C	0.45	0.039575
4	RSAD2	Radical *S*-adenosyl methionine domain containing 2	0.57	0.025385
5	TMPRSS2|AK	Transmembrane protease, serine 2	0.57	0.024545
6	DSC1	Desmocollin 1	1.62	0.035139
7	PAPSS2	3′-phosphoadenosine 5′-phosphosulphate synthase 2	0.63	0.016613
8	KIAA0746	KIAA0746 protein	0.63	0.039536
9	USP1	Ubiquitin-specific peptidase 1	1.37	0.027314
10	IL6R	Interleukin 6 receptor	1.34	0.004456
11	CPM	Carboxypeptidase M	1.34	0.026291
12	UQCRC2	Ubiquinol-cytochrome c reductase core protein II	1.28	0.011865
13	QSER1	Glutamine- and serine-rich 1	1.24	0.012726
14	PYGL	Phosphorylase, glycogen; liver (Hers disease, glycogen storage disease type VI)	1.24	0.013103
15	RBM25	RNA-binding motif protein 25	1.21	0.030094
16	KLF13	Kruppel-like factor 13	1.20	0.023861
17	ZFP91	Zinc finger protein 91 homologue (mouse)	1.16	0.035614
18	C16orf74	Chromosome 16 open reading frame 74	1.15	0.033430
19	NEK1	NIMA (never in mitosis gene a)-related kinase 1	0.88	0.033341
20	CUTL1	Cut-like 1, CCAAT displacement protein (Drosophila)	1.12	0.039922
(c)				
1	HBA1|HBA2	Haemoglobin, α 1|haemoglobin, α 2	0.24	0.021738
2	HBA1|HBA2	Haemoglobin, α 1|haemoglobin, α 2	0.24	0.025006
3	CXCL13	Chemokine (C–X–C motif) ligand 13 (B-cell chemoattractant)	4.01	0.007572
4	HBA1|HBA2	Haemoglobin, α 1|haemoglobin, α 2	0.24	0.021137
5	HBA1|HBA2	haemoglobin, α 1|haemoglobin, α 2	0.25	0.022495
6	HBA1|HBA2	Haemoglobin, α 1|haemoglobin, α 2	0.25	0.019494
7	HBB	Haemoglobin, β	0.30	0.036112
8	PDZRN4	PDZ domain containing RING finger 4	3.13	0.013944
9	CXCL6	Chemokine (C–X–C motif) ligand 6 (granulocyte chemotactic protein 2)	3.12	0.005698
10	HBB	Haemoglobin, β	0.32	0.039498
11	HBA1|HBA2	Haemoglobin, α 1|haemoglobin, α 2	0.33	0.025507
12	RGS4	Regulator of G-protein signalling 4	2.99	0.013331
13	UBD	Ubiquitin D	2.81	0.004953
14	HBB	Haemoglobin, β	0.35	0.041222
15	CCL19	Chemokine (C–C motif) ligand 19	2.76	0.006895
16	SAA1	Serum amyloid A1	2.67	0.007016
17	MGC23985	Similar to AVLV472	0.38	0.014519
18	DCT	Dopachrome tautomerase (dopachrome δ-isomerase, tyrosine-related protein 2)	0.40	0.019126
19	ARG1	Arginase, liver	0.40	0.003743
20	RP1-14N1.3	Filaggrin 2	0.41	0.000253

S. cerevisiae, *Saccharomyces cerevisiae*.

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Table 2. Top differentially expressed probes mapping to annotated genes, during the *(a)* first 4 days, *(b)* fifth and 10th day, *(c)* last 5 days of gingivitis resolution, sorted according to descending absolute fold change

Rank	Gene	Description	Fold change	*p*-value
(a)				
1	ODAM	Odontogenic, ameloblast asssociated	0.12	0.00164
2	HBA1|HBA2	Haemoglobin, α 1|haemoglobin, α 2	6.36	0.00429
3	HBA1|HBA2	Haemoglobin, α 1|haemoglobin, α 2	6.03	0.003739
4	HBA1|HBA2	Haemoglobin, α 1|haemoglobin, α 2	5.81	0.003716
5	HBA1|HBA2	Haemoglobin, α 1|haemoglobin, α 2	5.74	0.003961
6	HBA1|HBA2	Haemoglobin, α 1|haemoglobin, α 2	5.66	0.003054
7	C4orf26	Chromosome 4 open reading frame 26	0.18	0.00814
8	HBA1|HBA2	Haemoglobin, α 1|haemoglobin, α 2	4.92	0.004938
9	HBB	Haemoglobin, β	4.83	0.006822
10	HBB	Haemoglobin, β	4.47	0.005677
11	HBB	Haemoglobin, β	4.02	0.006566
12	SFRP4	Secreted frizzled-related protein 4	0.25	0.013203
13	RGS4	Regulator of G-protein signalling 4	0.26	0.000218
14	MS4A1	Membrane-spanning 4-domains, subfamily A, member 1	0.28	0.002695
15	PPBP	Pro-platelet basic protein (chemokine (C–X–C motif) ligand 7)	3.43	0.001211
16	CXCL1	Chemokine (C–X–C motif) ligand 1 (melanoma growth stimulating activity, α)	0.30	0.001902
17	PDZRN4	PDZ domain containing RING finger 4	0.32	0.001407
18	TNFRSF17	Tumour necrosis factor receptor superfamily, member 17	0.32	0.001979
19	MMP13	Matrix metallopeptidase 13 (collagenase 3)	0.33	0.029987
20	OGN	Osteoglycin (osteoinductive factor, mimecan)	0.33	0.011285
(b)				
1	C4orf26	Chromosome 4 open reading frame 26	5.82	0.046614
2	ODAM	Odontogenic, ameloblast asssociated	3.14	0.016792
3	CXCL11	Chemokine (C–X–C motif) ligand 11	2.95	0.019572
4	CXCL1	Chemokine (C–X–C motif) ligand 1 (melanoma growth stimulating activity, α)	0.38	0.030986
5	RP1-14N1.3	Filaggrin 2	0.47	0.02587
6	HAL	Histidine ammonia-lyase	2.07	0.002678
7	HTR3A	5-hydroxytryptamine (serotonin) receptor 3A	2.05	0.002189
8	ANXA9	Annexin A9	0.49	0.000984
9	UGT1A10|UG	UDP glucuronosyltransferase 1 family, polypeptide A10|UDP glucuronosyltransferase 1 family, polypept	0.49	0.019797
10	MMP13	Matrix metallopeptidase 13 (collagenase 3)	0.49	0.023574
11	S100P	S100 calcium-binding protein P	0.50	0.028185
12	AADAC	Arylacetamide deacetylase (esterase)	0.50	0.029201
13	XDH	Xanthine dehydrogenase	1.94	0.018272
14	TGM2	Transglutaminase 2 (C polypeptide, protein-glutamine-γ-glutamyltransferase)	1.90	0.036086
15	ISL1	ISL1 transcription factor, LIM/homeodomain, (islet-1)	1.87	0.000125
16	CXCL9	Chemokine (C–X–C motif) ligand 9	0.54	0.014364
17	POF1B|AK12	Premature ovarian failure, 1B	1.84	0.003542
18	LAMC2	Laminin, γ 2	0.54	0.033858
19	NRCAM	Neuronal cell adhesion molecule	1.82	0.011176
20	Placenta-specific 8	Placenta-specific 8	1.70	0.024099
(c)				
1	FOS	v-fos FBJ murine osteosarcoma viral oncogene homologue	3.19	0.02017
2	FOSB	FBJ murine osteosarcoma viral oncogene homologue B	3.08	0.040542
3	EGR1	Early growth response 1	2.83	0.005859
4	EGR1	Early growth response 1	2.71	0.004425
5	PTGS2	Prostaglandin-endoperoxide synthase 2 (prostaglandin G/H synthase and cyclooxygenase)	2.24	0.004138
6	ATF3	Activating transcription factor 3	2.09	0.026379
7	RFX2	Regulatory factor X, 2 (influences HLA class II expression)	1.98	0.037945
8	HBD	Haemoglobin, δ	1.94	0.013261
9	HBG1|HBG2	Haemoglobin, γ Alhemoglobin, γ G	1.90	0.036859
10	RGS1	Regulator of G-protein signalling 1	1.81	0.034072
11	NR4A2	Nuclear receptor subfamily 4, group A, member 2	1.79	0.002562
12	NR4A2	Nuclear receptor subfamily 4, group A, member 2	1.75	0.004211
13	RNASE7	Ribonuclease, RNase A family, 7	1.73	0.045338
14	DUSP1	Dual-specificity phosphatase 1	1.69	0.00139
15	SAMD4A	Sterile α motif domain containing 4A	0.60	0.00811
16	CDSN	Corneodesmosin	1.64	0.010418
17	EREG	Epiregulin	1.64	0.002051
18	CD55|AX772	CD55 molecule, decay accelerating factor for complement (Cromer blood group)	1.58	0.013212
19	NR4A2	Nuclear receptor subfamily 4, group A, member 2	1.58	0.002423
20	MNDA	Myeloid cell nuclear differentiation antigen	1.56	0.03622

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Table 3. Gene ontology analysis: gingivitis induction phase

Time point	Pathway	# Genes in pathway; % regulated	Impact factor	*p*-value
D7/D0	Leucocyte transendothelial migration	119; 19%	172.1	3.10E-73
	Cell adhesion molecules	116; 21%	116.3	3.44E-49
	Adherens junction	76; 32%	47.1	1.73E-19
	Huntington's disease	171; 39%	27.1	4.57E-11
	Ribosome	80; 42%	25.1	3.36E-10
D14/D7	Leucocyte transendothelial migration	114; 15%	325.4	1.52E-139
	Cell adhesion molecules	127; 17%	177.9	9.12E-76
	Phosphatidylinositol signalling system	76; 14%	15.6	2.72E-06
	Tight junction	131; 23%	8.6	0.00169301
	MAPK signalling pathway	266; 20%	8.1	0.00260222
D21/D14	Antigen processing and presentation	79; 37%	102.3	3.75E-43
	Cell adhesion molecules	127; 46%	77.4	1.85E-32
	Leucocyte transendothelial migration	114; 42%	51.5	2.29E-21
	Adherens junction	76; 29%	19.2	9.44E-08
	Allograft rejection	33; 55%	15.1	4.33E-06

MAPK, mitogen-activated protein kinase.

Table 4. Gene ontology analysis: gingivitis resolution phase

Time point	Pathway	# Genes in pathway; % regulated	Impact factor	*p*-value
D25/D21	Antigen processing and presentation	89; 33%	106.2	7.76E-45
	Leucocyte transendothelial migration	119; 35%	53.2	4.17E-22
	Cell adhesion molecules	134; 41%	42.7	1.25E-17
	Adherens junction	78; 29%	25.7	1.77E-10
	Phosphatidylinositol signalling system	76; 41%	13.9	1.42E-05
D30/D25	Leucocyte transendothelial migration	119; 24%	69.9	3.08E-29
	Adherens junction	78; 38%	29.3	5.82E-12
	Cell adhesion molecules	134; 22%	29.1	6.98E-12
	Antigen processing and presentation	89; 27%	26.6	7.94E-11
	Ubiquitin-mediated proteolysis	138; 40%	23.1	2.30E-09
D35/D30	Adherens junction	78; 14%	19.4	7.57E-08
	Circadian rhythm	13; 31%	16.6	1.04E-06
	MAPK signalling pathway	272; 16%	8.8	0.001438
	Phosphatidylinositol signalling system	76; 14%	6.8	0.008687
	Focal adhesion	203; 15%	6.8	0.008833

MAPK, mitogen-activated protein kinase.

week. Antigen processing and presentation and leucocyte transendothelial migration were the top differentially regulated pathways immediately after debridement and re-institution of oral hygiene, followed by cell adhesion molecules. Leucocyte transendothelial migration was still strongly regulated during the next 5 days of gingivitis resolution, but all other differentially regulated ontology groups had substantially lower impact factors.

To underscore the distinction between differential regulation on the pathway level and that on the individual gene level, we illustrate in Fig. 3 the within-pathway expression dynamics in a single ontology group (cell adhesion molecules) over time. The pathway was more strongly regulated during the induction phase (impact factor range 116.3 and 77.4) than in the resolution phase (impact factor range 42.7–1.7). Individual genes that were upregulated (red colour), downregulated (blue colour) or unchanged (green colour) are depicted. It is apparent that the direction of differential regulation within this pathway shifted signifiantly over time: for example, after a relatively uneventful second week of gingivitis induction (panel b), there was an obvious upregulation in multiple genes involved in antigen presentation and T-cell and B-cell signalling (c). In contrast, the first days of gingivitis resolution were characterized by extensive downregulation of multiple genes in this pathway.

Discussion

In this study, we used the experimental gingivitis model and whole-genome microarray technology to study the gingival tissue transcriptomic profiles during the induction and resolution of plaque-induced inflammation in a prospective longitudinal manner. To date, there is only a single report available in the literature that has adopted a similar approach to the study of the pathobiology of the reversible gingival lesion: recently, Offenbacher et al. (2009b) used an identical microarray platform and presented transcriptomic data from 14 participants in an experimental gingivitis study. Given the uniqueness of the published and the current data set, we briefly summarize some key points in the design of the two studies that are important in the comparative assessment and interpretation of their findings.

First, although experimental gingival inflammation in the Offenbacher and colleagues report was induced over a 4-week period, as compared with 3 weeks in the classic Löe et al. (1965) protocol as well as in the present study, the level of clinical inflammation reached at the peak of gingivitis induction at 28 days was less pronounced than the one observed in our study a week earlier, i.e., at 21 days. Specifically, GI in the Offenbacher and colleagues study increased from an average of 0.78 at baseline to 1.34 at 28 days, returning to 0.83 at the end of the resolution phase 1 week later. As shown by our data, our participants displayed almost absolute periodontal health at baseline, reflected by an average GI at the experimental sites of 0.1, reached a mean GI of 1.6 and 1.8 at 21 days in the induction and resolution groups, respectively, and returned to an average GI of 0.7 2 weeks after prophylaxis. These comparisons are admittedly crude as they are based on average values of categorical indices. Nevertheless, it is notable that 6 of the 20 participants in our study did not develop gingival inflammation beyond a GI score of 1 at day 21, consistent with the earlier documented heterogeneity in the clinical inflammatory response during experimental gingivitis (Tatakis & Trombelli 2004, Trombelli et al. 2008), possibly reflecting the lack of full compliance as well. Second, a more important difference between the two studies from a design perspective is the number of gingival tissue samples harvested from each participant and the time interval between the consecutive biopsies. In the Offenbacher and colleagues study, gingival tissue samples were obtained from all subjects on three occasions

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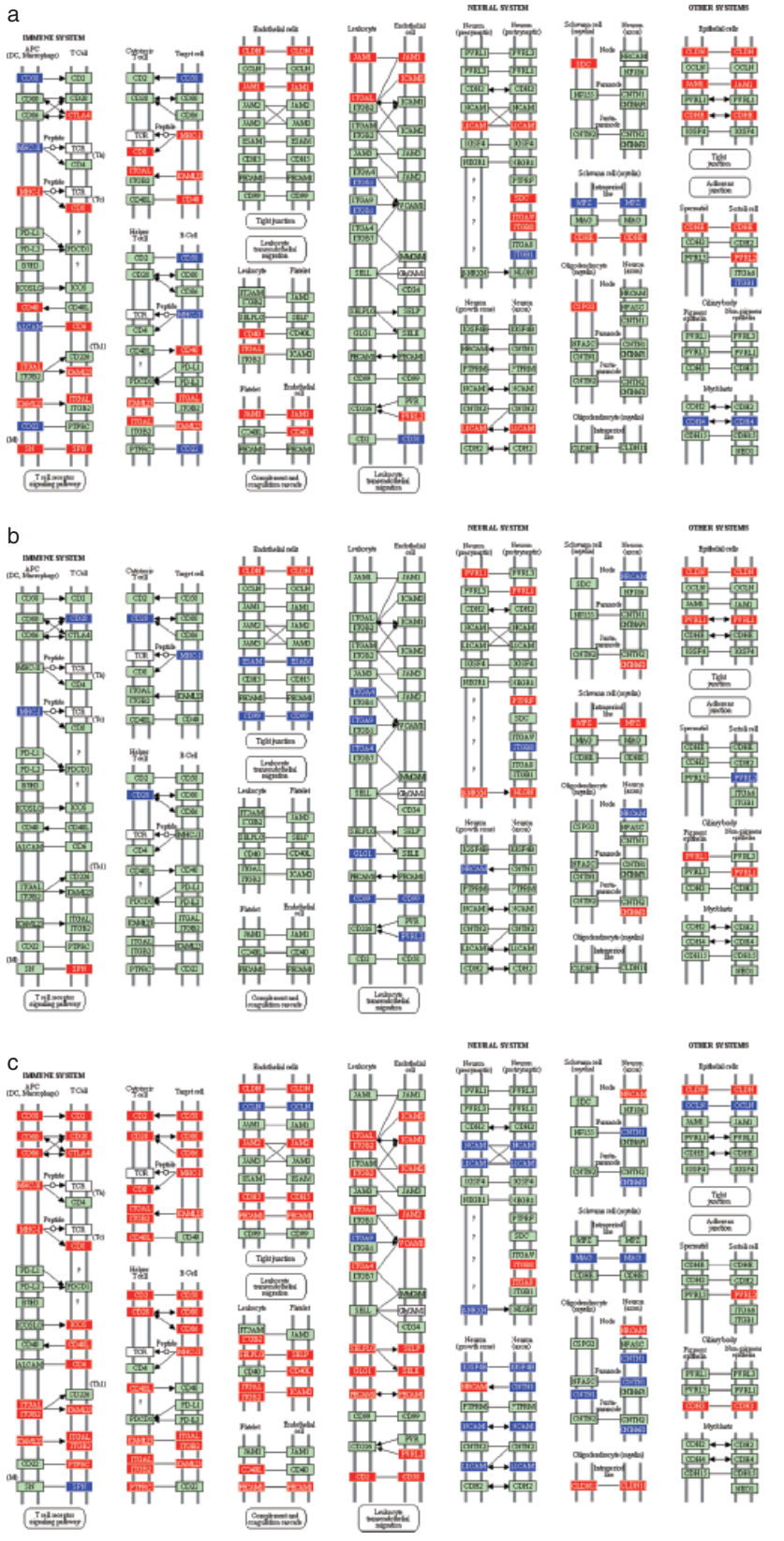

Fig. 3. Graphic illustration of the ''cell adhesion molecules'' pathway during the first (a), second (b) and third (c) week of gingivitis induction, as well as during the first 4 (d), subsequent 5 (e) and final 5 (f) days of gingivitis resolution. Genes depicted in red are upregulated in the latter *versus* the former time point, genes in blue are downregulated and genes in green are unchanged at the $p < 0.05$ significance level.

(baseline, Day 28 and Day 35). In the present study, we examined gingival tissue transcriptomes at four time points 1 week apart in the induction phase and at four time points 5 days apart in the resolution phase. To minimize the number of soft tissue samples that were obtained from each participant, we inevitably had to study the induction and resolution of gingivitis in two different groups of volunteers, comprising 10 individuals each. The significance of the availability of tissue from multiple time points within each phase is underscored by the data presented in Fig. 2: Thus, the number of up- or downregulated probes by at least 1.5-fold between day 21 and baseline was 184, and yet twice as many (373 probes) were differentially regulated within the third week of induction alone. Likewise, 470 probes were differentially regulated within the first 4 days post-intervention, whereas only 367 genes appeared to be differentially regulated between the end of the resolution period and the peak of gingivitis (Day 35 *versus* Day 21). Thus, our data suggest that the differential regulation of genes in the tissues over the course of gingivitis is not an additive, cumulative process that closely parallels the development of clinical inflammation but varies significantly among different time points within the 5-week experimental protocol. Thus, the assessment of gene expression at multiple time points within the induction and resolution phase rather than a three time point, ''snapshot'' description over the entire experimental period (Offenbacher et al. 2009b), appears to better reflect the kinetics of sequential gene expression, although this approach necessitated the involvement of separate groups of individuals in the two phases.

The number of differentially expressed probes by >1.5-fold (Fig. 2), as well the number of probes that were significantly ($p < 0.05$) regulated between any two time points irrespective of fold change, suggest that the two most ''eventful'' time periods with respect to transcriptomic activation during the course of experimental gingivitis are the third week of gingivitis induction and the first 4 days of gingivitis resolution. These observations are in agreement with our current understanding of the biological events occurring at the plaque biofilm/gingival tissue interface. Indeed, it makes biological sense that a certain level of maturation of the dental plaque is required to elicit the apparent robust

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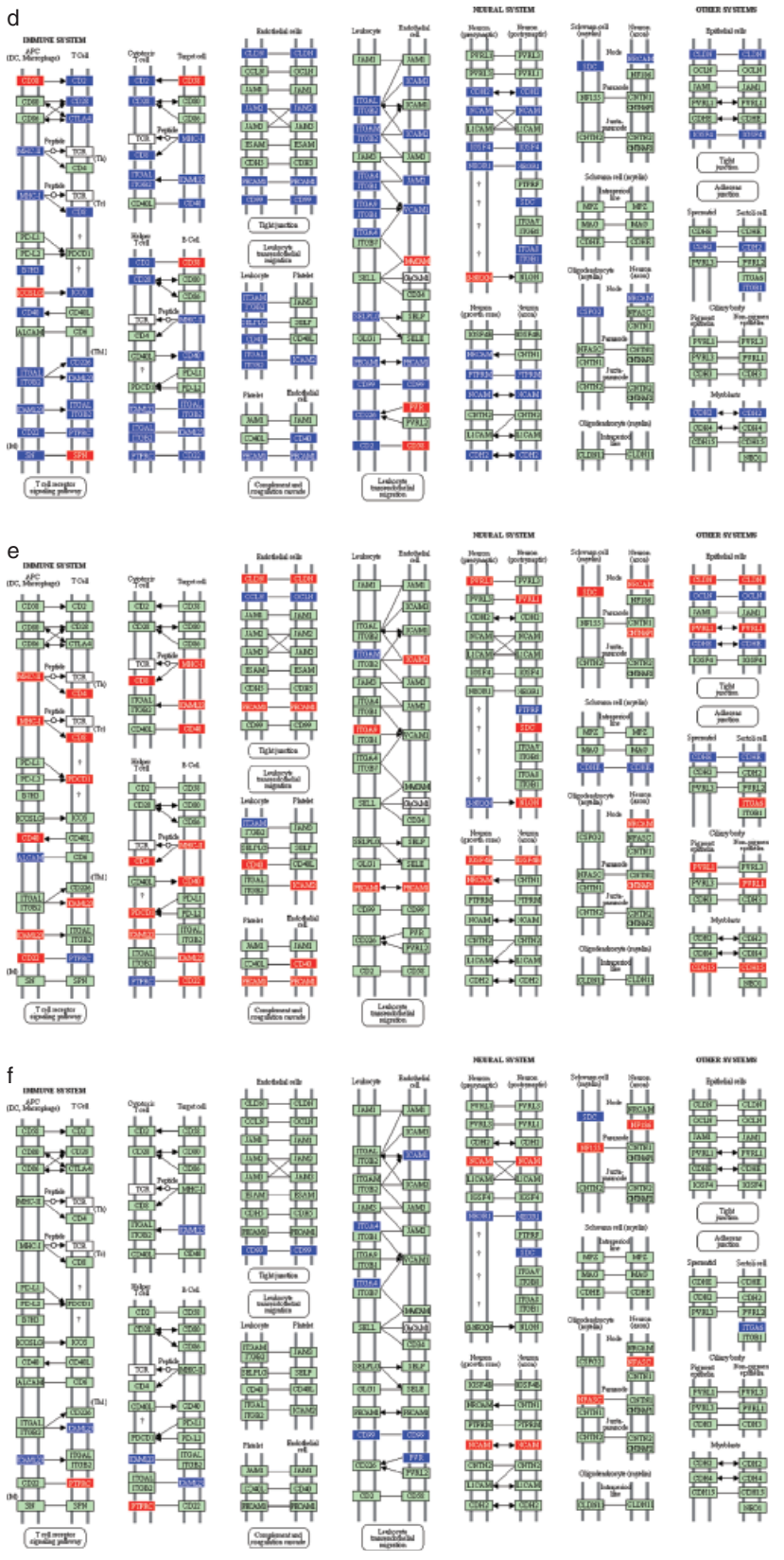

Fig. 3. Continued.

mobilization of the adaptive immune response that occurred during the final week of gingivitis induction. Likewise, the abrupt dispersion of the established biofilm achieved through prophylaxis, likely in combination with an instrumentation-induced mechanical stimulation of the tissues, triggered an immediate and rather profound transcriptomic response.

A closer look at the top differentially regulated genes in the first week of gingivitis induction (Table 1a) identified cysteine-rich secretory protein 3 (CRISP3), an innate host defence gene coding for a protein that is present in peroxidase-negative granules of neutrophils and in exocrine secretions (Udby et al. 2002) to be upregulated by 5.54-fold. Other strongly upregulated genes included MS4A1 (CD20, membrane-spanning four-domains, subfamily A, member 1), a gene that encodes a B-lymphocyte surface molecule involved in the development and differentiation of B-cells into plasma cells (Petrie & Deans 2002) upregulated by 3.24-fold, and CD177, a neutrophil-specific, heterophilic-binding partner of the platelet endothelial cell adhesion molecule 1 (PECAM-1) (Sachs et al. 2007), upregulated by 2.54-fold. Interestingly, CRISP3 was also the top differentially expressed gene during the second week of gingivitis (Table 1b), but this time downregulated by approximately threefold. In week 3 (Table 1c), multiple haemoglobin α 1 and α 2 probe sets were significantly downregulated, while the top upregulated gene (by fourfold) was CXCL13, a CXC chemokine that promotes the migration of B lymphocytes (Stachowiak et al. 2006), followed by CXCL6, a granulocyte chemoattractant protein recently shown by our group to also be significantly upregulated in periodontitis lesions (Kebschull et al. 2009). CCL19, a CC motif chemokine involved in lymphocyte and dendritic cell trafficking (Leick et al. 2010), was also upregulated by approximately threefold. Multiple probes associated with natural killer (NK) cell function were also differentially regulated during the third week of induction, including killer cell lectin-like receptors (KCLLR) B1, C1 and K1 (with fold changes of 1.72, 1.60 and 1.59, respectively) as well as granzymes A, B and K (with fold changes of 1.70, 1.69 and 1.62, respectively). These findings are in accordance with earlier histologic observations (Wynne et al. 1986) demonstrating a gradual increase in the number of NK cells during the course of experimental gingivitis. They are of particular interest as NK cells represent a link between a bacterially induced immune response and an auto-immune component that has been suggested to play a

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role in the pathobiology of periodontitis (Yamazaki et al. 2001).

Conversely, several haemoglobin-encoding genes were significantly upregulated during the first 4 days after prophylaxis and re-institution of oral hygiene (Table 2a), as was pro-platelet basic protein (PPBP), a CXC chemokine family member that is part of the secretory antimicrobial arsenal of the human monocytes (Schaffner et al. 2004). In contrast, CXCL1 was found to be downregulated by approximately threefold, as was TNFRSF17 (tumour necrosis factor receptor superfamily, member 17), a receptor preferentially expressed by mature B-lymphocytes, which, when bound to its ligand TNFSF13B, mediates NF-κB and MAPK8/JNK activation (Hatzoglou et al. 2000). Additional genes that were found to be downregulated by approximately threefold during the first days of gingivitis resolution included matrix metallopeptidase 13 (MMP-13), and osteoglycin (OGN), a proteoglycan with osteoinductive capabilities (Kukita et al. 1990). Interestingly, CXCL1 and MMP-13 were further downregulated during the next 5-day period (Table 2b). The last 5 days of the gingivitis resolution (Table 3b) were characterized by the induction of several genes involved in differentiation, including FOS, FOSB, early growth response 1 (EGR1), prostaglandin-endoperoxide synthase 2 (PTGS2) and activating transcription factor 3 (ATF3). The first two belong to the four-member FOS gene family that encodes proteins regulating proliferation, differentiation and transformation (Durchdewald et al. 2009). EGR1 is a nuclear protein that acts as a transcriptional regulator with a role in differentiation and mitogenesis (Braddock 2001). PTGS2, also known as cyclooxygenase 2, is a key enzyme in prostanoid biosynthesis, and ATF3 is a mammalian activation transcription factor (Thompson et al. 2009). Collectively, these top upregulated proteins in the last phase of the resolution period may reflect the ongoing healing processes in the gingival tissues.

Gene ontology analyses identified consortia of genes that broadly orchestrate the soft tissue responses. As shown in Table 3, ''leucocyte transendothelial migration'' and ''cell adhesion'', the two pathways with most significant regulation in both the first and the second week of induction, were more strongly regulated during the second than the first week. ''Antigen processing and presentation'' was the strongest regulated gene ontology group during the final week of induction, indicating a robust mobilization of the adaptive immune response. The ''antigen processing and presentation'' pathway was in fact stronger regulated during the first 4 days of gingivitis resolution than in the final week of induction (impact factor 106.2), possibly due to the inoculation of the host with bacteria and their products in conjunction with mechanical prophylaxis. These transcriptomic findings are largely in agreement with earlier histologic observations of the initial and early gingival lesions, first described in detail by Page & Schroeder (1976) primarily based on animal experiments, but also with subsequent human histo-morphometric studies (Seymour et al. 1983, Brecx et al. 1987, Moughal et al. 1992). Nevertheless, it must be recognized that considerable heterogeneity in the histological features of experimental gingivitis lesions has been reported in the literature. For example, data by Kinane et al. (1991) on human gingival biopsies obtained at baseline and after 7, 14 and 21 days of experimental gingivitis demonstrated that infiltration by PMN cells, T-cells and HLA-DR+antigen presenting cells, as well as expression of adhesion molecules ELAM-1 and ICAM-1, all peaked at Day 7 and gradually subsided through Day 21. In contrast, the earlier work of Seymour et al. (1983) demonstrated that approximately 70% of the cellular infiltrate throughout the course of experimental gingivitis consisted of T-lymphocytes, and that this proportion remained fairly constant over time despite an increase in infiltrate size. Our gene ontology data do not corroborate the finding obtained by Offenbacher et al. (2009b) of a substantial transient activation of genes involved in neural processes during experimental gingivitis, but differences in the time points of tissue harvesting may partly account for this discrepancy. An attempt to carry out a direct comparison of the probe sets that were statistically ($p<0.05$) differentially regulated at opposite directions (''up/down'', or ''down/up'' genes) during induction (Day 28 *versus* baseline) and resolution (Day 35 *versus* Day 28) in the Offenbacher et al. (2009b) dataset to those with similar differential regulation at the best corresponding time points in our data set (Day 21 *versus* baseline, and Day 30 *versus* Day 21, respectively) identified a limited number of transiently regulated genes that were common in both datasets: genes upregulated in induction and downregulated in resolution included CCL5 (RANTES), a CC cytokine that is chemoattractant for blood monocytes, memory T-helper cells and eosinophiles (Levy 2009); PYHIN1 (pyrin and HIN domain family, member 1), a primarily nuclear protein involved in transcriptional regulation of genes affecting cell cycle control, differentiation and apoptosis (Ding et al. 2006); granzyme A (GZMA), a cytotoxic T-cell and natural killer cell-specific serine esterase (Grossman et al. 2004); CD96, a membrane protein involved in antigen presentation and in adhesive interactions between activated T and NK cells (Fuchs et al. 2004); Adducing 3 (ADD3), a protein involved in the assembly of spectrin-actin networks and cell-to-cell contact in epithelial tissues (Kaiser et al. 1989); and Toll-like receptor (TLR) 7, one of the intra-cellular, nucleic-acid-sensing TLRs (Krieg & Vollmer 2007), whose differential expression during gingivitis likely reflects host cell activation in response to internalized bacteria. Probes common to both data sets that were downregulated during induction and upregulated during resolution included TMEM16A (anoctamin 1), involved in epithelial volume-regulated chloride channels with potential function in proliferation and apoptosis (Almaca et al. 2009) and genes coding for the matrix proteins lamin A/C (LMNA) (Wagner & Krohne 2007) and chondroitin sulphate proteoglycan 4 (CSPG4; Lorber 2006).

We acknowledge some important limitations of the current work. First, the transcriptomic data have been derived from a relatively small sample of young volunteers, and it is unlikely that they capture the full extent of the variability in gene expression profiles in experimental gingivitis across individuals or age groups. In addition, the limited sample size did not allow for full adjustments for multiple comparisons in the identification of significantly regulated probes, similar to the published report (Offenbacher et al. 2009b). Second, it must be recognized that longitudinal changes in gingival inflammation and consequently in the gingival transcriptomic profiles cannot be exclusively attributed to plaque accumulation or biofilm dispersion, but are

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also influenced by additional exposures such as hormonal fluxes in females and dietary effects. Ideally, these could have been accounted for by studying over time gingival units not subjected to experimental gingivitis, but a study design requiring serial harvesting of additional tissue samples was not feasible. Third, due to the exploratory and descriptive nature of this work, we have not yet carried out independent verification of specific genes by a second, mRNA-based method, such as real-time RT-PCR. Lastly, verification steps at the protein level need to be performed, either on tissue extracts or on gingival GCF samples. We have indeed obtained GCF samples over time from the crevices adjacent to the harvested tissue papillae, and are carrying out high-throughput proteomic analyses to examine the extent to which gingival tissue mRNA sequences translate into GCF proteins. In the future, we envision that it will be possible to evaluate the effects of adjunctive pharmacological therapies on the gingival tissue transcriptome during the induction and resolution of gingivitis against the present data.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. Periodontal microbiota during the induction (left panel) and resolution (right panel) of experimental gingivitis. Bars represent mean counts $\times$ 104. Note the difference in the y-axis scale among the different microbial species.

Table S1. Differentially regulated probes ($p < 0.05$) during the first week of induction (Day 7/Baseline), sorted according to descending absolute change.

Table S2. Differentially regulated probes ($p < 0.05$) during the second week of induction (Day 14/Day 7), sorted according to descending absolute change.

Table S3. Differentially regulated probes ($p < 0.05$) during the third week of induction (Day 21/Day 14), sorted according to descending absolute change.

Table S4. Differentially regulated probes ($p < 0.05$) during the first four days of resolution (Day 25/Day 21), sorted according to descending absolute change.

Table S5. Differentially regulated probes ($p < 0.05$) during the forth to ninth day of resolution (Day 30/Day 25), sorted according to descending absolute change.

Table S6. Differentially regulated probes ($p < 0.05$) during the final 5 days of resolution (Day 30/Day 25), sorted according to descending absolute change.

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Clinical Relevance

Scientific rationale for the study: The study of tissue responses in experimental gingivitis has largely focused on the identification of cell populations using immunohistochemistry or on the assessment of the levels of selected proteins in the GCF. Data on the sequential activation of genes in the host tissues during the induction and resolution of gingival inflammation are sparse.

Principal findings: Our data indicate that the differential expression of genes in the gingival tissues reaches its peak during the third week of experimental gingivitis and during the first 4 days of re-institution of oral hygiene. Our work identifies networks of genes that orchestrate these tissue responses.

Practical implications: Our work furthers our understanding of the gingival responses to plaque accumulation and plaque control, and can serve as a basis for a comparison of the effects of adjunctive pharmacological agents in future studies.

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